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Metabolic background determines the importance of *NOS3* polymorphisms in restenosis after percutaneous coronary intervention: A study in patients with and without the metabolic syndrome

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Abstract. Variation in the *NOS3* gene has been related to the development of restenosis. The Glu298Asp polymorphism has previously been investigated for its effect on NO levels and the development of restenosis. However, the variability of findings gave rise to the hypothesis that the functional significance of this polymorphism may only become manifest under conditions of endothelial dysfunction. Since patients with the metabolic syndrome are known to have endothelial dysfunction, we aimed to investigate if the significance of *NOS3* polymorphisms may depend on the presence of the metabolic syndrome.

We examined the impact of the -949 A/G, the -716 C/T and the Glu298Asp polymorphisms in the *NOS3* gene on the risk of clinical restenosis in a previously described subpopulation of the GENDER-study, a multicenter prospective study design that enrolled consecutive patients after successful PCI. This subpopulation consisted of 901 patients of whom sufficient data were available to establish absence or presence of the metabolic syndrome. Of these patients, 448 had the metabolic syndrome. Clinical restenosis, defined as target vessel revascularization (TVR), was the primary endpoint.

We demonstrated that the minor -949G, -716T and 298Asp alleles were associated with a significantly increased risk of TVR in patients with the metabolic syndrome (HR: 1.58 [95%CI: 1.04–2.40], HR: 1.95 [95%CI: 1.02–3.70] and HR: 1.67 [95%CI: 1.09–2.54], respectively). In the group without the metabolic syndrome we observed no association between the three polymorphisms and TVR.

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We suggest that the metabolic alterations present in patients with the metabolic syndrome influence the functional significance of these polymorphisms and increase the susceptibility of carriers of one of these variations in the *NOS3* gene to develop restenosis after PCI.

Keywords: Restenosis, percutaneous coronary intervention, metabolic syndrome, nitric oxide synthase, polymorphisms

1. Introduction

The frequent occurrence of restenosis is a major limitation to percutaneous coronary intervention (PCI) in patients with coronary artery disease. Patients at increased risk for restenosis could benefit from alternative treatment options such as drug-eluting stents or coronary artery bypass surgery. To be able to stratify patients with regard to their risk of developing restenosis, it is important to determine both inherited and clinical factors influencing this complex and multifactorial process.

Nitric oxide (NO) is a vasodilatory molecule known to inhibit adhesion of platelets [27] and leukocytes [16] to the vessel wall and to decrease proliferation and migration of vascular smooth muscle cells (VSMCs) [9], which are all processes known to play an important role in the development of restenosis [3]. Due to its favourable actions in these processes, NO is likely to play a protective role in the formation of restenosis. Restenosis has already been related to alterations in nitric oxide synthase (NOS) expression [24] and impaired NO-dependent endothelial vasodilation [43]. In support of these observations, Piatti et al. showed a lack of NO release after an oral glucose tolerance test (OGTT) in patients with restenosis [26]. Since endothelial NOS catalyses the formation of NO from L-arginine in endothelial cells, polymorphisms in the *NOS3* gene could influence NO formation and thereby play a role in the process of restenosis. The Glu298Asp polymorphism in the *NOS3* gene has previously been investigated. However, data on its influence on NO levels and the development of restenosis are still conflicting. Sofowora et al. suggested that the functional significance of this polymorphism may only become manifest under conditions of endothelial dysfunction [32]. Since endothelial dysfunction is an important characteristic of the metabolic syndrome, we hypothesize that the metabolic alterations present in patients with the metabolic syndrome may influence the functional significance of *NOS3* polymorphisms. Therefore, the aim of this study was to examine whether the -949 A/G, the -716 C/T and the Glu298Asp polymorphisms in the *NOS3* gene are related to the risk of restenosis after PCI in patients with

and without the metabolic syndrome. The GENDER protocol was designed to allow separate analyses in a prespecified subgroup of patients with the metabolic syndrome.

2. Patients and methods

The present study population has been described previously [4]. In brief, the GENetic DEterminants of Restenosis project (GENDER) was designed to study the association between genetic polymorphisms and clinical restenosis. Patients were eligible for inclusion if they were successfully treated for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI. Patients treated for acute ST elevation myocardial infarction were excluded. All patients were treated in 4 of the 13 referral centers for interventional cardiology in the Netherlands. The overall inclusion period lasted from March 1999 until June 2001. We determined the lipid profile (serum triglycerides, serum total cholesterol, serum HDL-cholesterol) and fasting serum glucose in a subpopulation of patients of whom plasma was collected. The study protocol conforms to the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from each participant before the PCI procedure.

2.1. PCI procedure

Standard angioplasty and stent placement were performed by experienced operators using a radial or femoral approach. Before the procedure, patients received aspirin 300 mg and heparin 7500 IU. The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, was at the discretion of the operator. In case of stent implantation, patients received either ticlopidin or clopidogrel for at least one month following the procedure depending on local practice. During the study, no drug-eluting stents were used.

2.2. Follow-up and study endpoints

Follow-up lasted at least nine months, except when a coronary event occurred. Patients were either seen in the outpatient clinic or contacted by telephone. Restenosis was defined by target vessel revascularization (TVR), either by PCI or coronary artery bypass grafting (CABG). An independent clinical events committee adjudicated the clinical events.

Events occurring within the first month were excluded from the analysis, since these events were attributable mainly to sub-acute stent thrombosis or occluding dissections, and less likely to restenosis. Data were collected with standardized case-report forms that were completed by the research coordinator at each site. Representatives from the data-coordinating center monitored all sites.

2.3. Genotyping

Blood was collected in EDTA tubes at baseline and DNA was extracted following standard procedures. The NOS3 -949 A/G (rs1800779), -716 C/T (rs3918226) and the 5219 G/T (rs1799983) polymorphism, resulting in the amino acid substitution Glu298Asp, were determined by a validated multilocus genotyping assay (Roche Molecular Systems) [6]. The Glu298Asp polymorphism has already been implicated in coronary artery disease [2] and restenosis [12, 35]. The -949 A/G polymorphism was selected on the basis of its involvement in atherosclerotic disease [15] and the -716 C/T polymorphism is located between the cAMP regulatory element (nt -726 to -732) and an activator protein-1 binding domain (nt -655 to -661) [39], which suggests a potential involvement in gene regulation.

In short, each DNA sample was amplified in a multiple polymerase chain reaction (PCR) using biotinylated primers. The PCR product pool was then hybridized to a matching panel of sequence-specific oligonucleotide probes, immobilized in a linear array on nylon membrane strips. A colorimetric detection method based on incubation with streptavidin-horseradish peroxidase conjugate, using hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine as substrates, was used. Operators blinded to restenosis status performed genotyping.

To confirm genotype assignments, the PCR-procedure was performed in replicate on 10% of the samples. Two independent observers carried out scoring. Disagreements (<1%) were resolved by further joint reading, and when necessary, genotyping was repeated.

2.4. Definitions

Patients were defined as having metabolic syndrome by presence of three or more of the following criteria: 1) triglyceride level ≥ 1.7 mmol/l (150 mg/dl); 2) HDL cholesterol level < 1.04 mmol/l (40 mg/dl) in men and < 1.3 mmol/l (50 mg/dl) in women; 3) systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg; 4) obesity, defined as BMI > 28.8 kg/m², which was equivalent to a waist circumference of 102 cm in a cross-sectional study and similar to the BMI value (28.2 kg/m²) calculated in a regression of BMI on waist circumference in a large population of Scottish men [18,30] and 5) fasting glucose level ≥ 5.55 mmol/l (100 mg/dl), above which patients have either pre-diabetes (impaired fasting glucose) or diabetes. This level was established by the American Diabetes Association and suggested as the new cut point for identifying the lower boundary to define an elevated glucose level as one criterion for the metabolic syndrome [1,10,14].

2.5. Statistical methodology

Allele frequencies were determined by gene counting, the 95% confidence intervals of the allele frequencies were calculated from sample allele frequencies, based on the approximation of the binominal and normal distributions in large sample sizes. Continuous variables are expressed as mean \pm standard deviation and were compared by means of an unpaired, two-sided *t* test. Discrete variables are expressed as counts or percentages and were compared with a chi-square test.

The association between each of the 3 polymorphisms and TVR was determined using a Cox proportional regression model. If less than 10 patients were homozygous for a particular allele, the homozygotes and heterozygotes were combined. Polymorphisms with independent prognostic value were selected in a multivariable regression model, also containing clinical and procedural risk factors.

To investigate possible additive effects of haplotypes of the NOS3 polymorphisms on TVR risk, we analyzed haplotype effects using the THESIAS program (www.genecanvas.org), which implements the stochastic-EM (Expectation–Maximization) algorithm and allows the use of Cox proportional hazards survival regression [38]. Statistical analyses were carried out using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

The characteristics of this patient sample have been described previously [4]. In summary, a total of 3,146 patients had complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9 months). Out of 3,146 patients 42 experienced an event in the first 30 days and were therefore excluded from further analysis, according to the protocol. In a previously described subpopulation of 901 patients [28], data were sufficient to establish absence or presence of metabolic syndrome. Of these patients, 448 (49.7%) had metabolic syndrome (≥ 3 of the 5 characteristics). The prevalence of metabolic syndrome characteristics in patients with and without the metabolic syndrome, as well as demographic, clinical and lesion characteristics, are presented in Table 1. The presence of one or more components of the metabolic syndrome was common in both sexes: 16.3% had one component, 29.6% had two components, 26.9% had three components, 15.9% had four components, and 7.0% had all five components. Except for the presence of diabetes, the prevalence of factors that were previously found to be related to restenosis did not differ between the two groups. However, patients with the metabolic syndrome had higher insulin levels and were more frequently treated with statins or insulin.

At follow-up 47 patients (10.5%) with the metabolic syndrome versus 45 (9.9%) without the metabolic syndrome underwent TVR. On multivariable Cox regression, there was no significant increase in risk of TVR in patients with the metabolic syndrome (HR: 1.03 [95%CI: 0.68–1.57]).

Genotyping of the -949 A/G, the -716 C/T and the Glu298Asp (G/T) polymorphisms was successful in 96.9%, 96.7% and 96.9% of patients with the metabolic syndrome and in 98.7%, 98.2% and 98.2% of those without the metabolic syndrome, respectively. Results of the remaining patients were missing due to lack of DNA or inconclusive genotyping. In both groups, the frequencies of the minor -949G, -716T and 298Asp alleles were 0.37, 0.09 and 0.31, respectively. The genotype distributions were consistent with Hardy-Weinberg equilibrium and did not differ significantly between patients with the metabolic syndrome and controls ($p > 0.05$).

Univariate Cox regression in patients with the metabolic syndrome revealed a significant association between the -949 A/G, the -716 C/T and the Glu298Asp polymorphisms and the rate of TVR (HR: 1.58 [95%CI: 1.04–2.40] $p = 0.034$, HR: 1.95 [95%CI: 1.02–3.70]

$p = 0.042$ and HR: 1.67 [95%CI: 1.09–2.54] $p = 0.018$, respectively) (Table 2).

TVR occurred more often in -949 GG homozygotes (18.2%) than in AG heterozygotes (11.0%) or AA homozygotes (7.6%). Since only one patient was homozygous for the -716T allele, we combined homozygous and heterozygous patients for this polymorphism. This combined group had an increased risk of TVR (17.6%) when compared to the group with the -716 CC genotype (9.2%). Homozygotes and heterozygotes for the 298Asp allele also had a higher risk of TVR (21.1% and 11.5%, respectively) when compared to the Glu/Glu genotype (7.8%).

In contrast, in patients without the metabolic syndrome, the -949 A/G, -716 C/T and the Glu298Asp polymorphisms were not associated with TVR (HR: 0.90 [95%CI: 0.57–1.43] $p = 0.65$, HR: 0.33 [95%CI: 0.10–1.08] $p = 0.067$ and HR: 1.02 [95%CI: 0.66–1.59] $p = 0.93$, respectively). For each polymorphism, TVR-risks were equal in the different genotype groups (Table 2).

After adjustment for patient and intervention-related characteristics that were previously described to be related to TVR risk, such as age, sex, hypertension, diabetes, current smoking, stenting, total occlusion and residual stenosis $> 20\%$, the -949 A/G, the -716 C/T and the Glu298Asp polymorphisms were predictors of TVR (HR: 1.55 [95%CI: 1.01–2.37] $p = 0.043$, HR: 2.05 [95%CI: 1.05–4.00] $p = 0.034$ and HR: 1.68 [95%CI: 1.09–2.59] $p = 0.019$, respectively).

As determined with haploview, the -949 A/G and -716 C/T polymorphisms revealed significant linkage disequilibrium (D' : 0.95) and were combined into haplotypes for further analysis. The Glu298Asp polymorphism was excluded from this analysis due to the lack of linkage disequilibrium between this polymorphism and the -949 A/G polymorphism (D' : 0.45). In both groups, frequencies of the -949A/-716C, -949G/-716C and -949G/-716T haplotypes were 0.62, 0.29 and 0.08, respectively. Due to its low frequency (0.007 in patients with the metabolic syndrome and 0.008 in patients without the metabolic syndrome), the -949A/-716T haplotype was excluded from the analysis. A haplotype-based risk analysis in patients with the metabolic syndrome revealed a significant association of the -949G/-716T haplotype with the risk for TVR when compared to -949A/-716C, the most frequent reference haplotype (HR: 2.89 [95%CI: 1.45–5.76], $p = 0.0026$ (Fig. 1)).

Table 1
Baseline and metabolic syndrome characteristics by metabolic syndrome status ($N = 901$)

	Metabolic syndrome		<i>P</i> value ^a
	Absent	Present	
<i>Baseline Characteristics:</i>	<i>N</i> = 453	<i>N</i> = 448	
Age (y \pm SD)	63 (\pm 10)	61 (\pm 11)	0.003
BMI (kg/m ² \pm SD)	25.4 (\pm 3.0)	28.7 (\pm 4.1)	< 0.001
Female sex (%)	127 (28)	131 (29)	0.69
Diabetes Mellitus (%)	32 (7)	108 (24)	< 0.001
Current smoking (%)	80 (18)	94 (21)	0.21
Stent placement (%)	376 (80)	373 (83)	0.92
Residual stenosis (%)	51 (11)	44 (10)	0.52
Total occlusion (%)	32 (7)	41 (9)	0.27
Total cholesterol (mmol/L, \pm SD)	4.91 (\pm 1.05)	5.01 (\pm 1.12)	0.32
Statins (%)	231 (51)	265 (59)	0.014
Insulin ^b (mU/l, IQR)	12 (9)	20 (20)	< 0.001
Insulin therapy (%)	13 (3)	32 (7)	0.003
<i>Metabolic Syndrome Characteristics</i>			
BMI > 28.8 kg/m ²	37 (8.2%)	207 (46.2%)	
Triglycerides \geq 1.7 mmol/L	128 (28.3%)	370 (82.6%)	
HDL cholesterol < 1.04 mmol/L men and < 1.3 mmol/L women	128 (28.3%)	324 (72.3%)	
Systolic BP \geq 130 and diastolic BP \geq 85 mm Hg	273 (60.3%)	383 (85.5%)	
Fasting glucose \geq 5.55 mmol/L	115 (25.4%)	329 (73.4%)	

^a p-value of the nonparametric Mann-Whitney test, or chi-square test.

^b Insulin levels are presented as median with interquartile range (IQR).

Table 2
Cox regression of NOS3 polymorphisms in association with TVR in patients with and without the metabolic syndrome

Metabolic syndrome	Polymorphisms	Number of patients	TVR (%)	HR (95%CI)
Present (<i>N</i> = 448)	-949 A/G			
	AA	170	7.6	1.58 (1.04–2.40)
	AG	209	11.0	
	GG	55	18.2	
	-716 C/T			
	CC	359	9.2	1.95 (1.02–3.70)
	CT	73	17.6	
	TT	1		
	Glu298Asp (G/T)			
	GG	204	7.8	1.67 (1.09–2.54)
	GT	192	11.5	
	TT	38	21.1	
Absent (<i>N</i> = 453)	-949 A/G			
	AA	167	10.8	0.90 (0.57–1.43)
	AG	226	8.4	
	GG	54	11.1	
	-716 C/T			
	CC	374	10.7	0.33 (0.10–1.08)
	CT	63	4.2	
	TT	8		
	Glu298Asp (G/T)			
	GG	219	10.0	1.02 (0.66–1.59)
	GT	174	8.0	
	TT	52	13.5	

4. Discussion

To test our hypothesis that the functional significance of NOS3 polymorphisms may depend on the presence

of the metabolic syndrome, we studied the relationship between these polymorphisms and the risk of restenosis in a subpopulation of patients with the metabolic syndrome that took part in a large follow-up study of

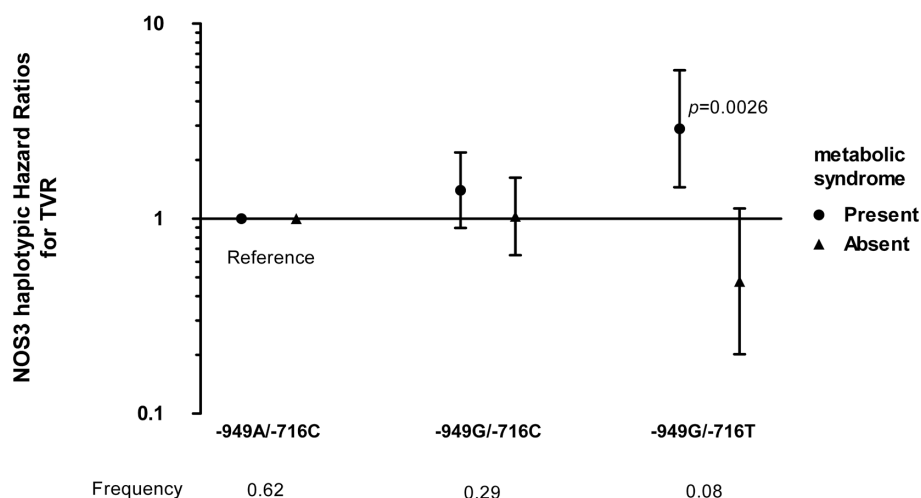


Fig. 1. *NOS3* haplotype effects on the risk for target vessel revascularization (TVR) in patients with (circles) and without (triangles) the metabolic syndrome. Hazard ratios by reference to the most frequent haplotype were calculated using the THESIAS software. The -949G/-716T promoter haplotype showed a strong association with TVR (HR: 2.89 [95%CI: 1.45–5.76], $p = 0.0026$), which indicates that both polymorphisms contribute separately to the risk of TVR.

unselected, consecutive patients undergoing PCI. We demonstrated that the -949G, -716T and 298Asp variants in the *NOS3* gene were associated with a highly increased risk of TVR after PCI in patients with the metabolic syndrome, but not in patients without the metabolic syndrome.

Of the -949 A/G, -716 C/T and Glu298Asp polymorphisms, only the Glu298Asp polymorphism has previously been investigated for its involvement in restenosis. Suzuki et al. have demonstrated that carriers of the 298Asp allele were at increased risk of developing angiographic restenosis after stent placement (OR: 3.9 [95%CI: 1.09–13.95], after multivariate analysis) [35]. However, their population was very small ($n = 85$) and consisted of relatively many patients with diabetes (27%). In support of their results, Gomma et al. demonstrated in a still relatively small and selected group of 205 stented patients, that possession of one or more alleles of the 298Asp variant was associated with an increased rate of angiographic restenosis after adjusting for clinical factors (OR: 2.47 [95% CI: 1.22–5.00]) [12]. However, Gorchakowa et al. could not confirm these findings and demonstrated in a similar study, with a much larger patient number ($n = 1850$), that rates of angiographic restenosis or TVR did not differ significantly between the Glu298Asp genotypes [13].

The mechanism by which this polymorphism would promote neointima formation remains subject to discussion. It was originally suggested that NO would protect against restenosis because of its inhibitory role in VSMC proliferation, adhesion of leukocytes to the

vessel wall and coagulation [9,16,27], processes known to be involved in restenosis [3]. In addition, NO has been shown to suppress the production of the potent vasoconstrictors endothelin and angiotensin II, that also induce VSMC proliferation [5,36]. Its role in restenosis was further established by the observation that L-arginine, the precursor to NO, decreases restenotic lesion formation in the rat [41], whereas L-NAME (N^G -nitro-L-arginine methyl ester), a non-selective NOS-inhibitor, stimulates neo-intimal hyperplasia in the rabbit after balloon angioplasty [17]. In humans, intramural administration of L-arginine has been shown to reduce neointimal hyperplasia at 6 months after stent placement [34]. Furthermore, patients with in-stent restenosis had impaired endothelial vasodilation and a lack of NO-release after an OGTT when compared to patients without restenosis [26].

Although several *in vitro* [7] and *in vivo* [32,40] studies showed reduced levels of NO in Glu298Asp homozygotes, data on the functional significance of this polymorphism are still conflicting. The 298Asp variant has been shown to result in protein with twofold less activity [42] and an increased susceptibility to proteolytic cleavage [37]. However, Sessa et al. demonstrated that the observed fragmentation was an artifact of sample preparation for gel electrophoresis [8] and several authors reported similar activity of *NOS3* in the different Glu298Asp genotypes [11,22]. Studies examining vasodilation in response to intrabrachial injection of acetylcholine produced conflicting results [29,31]. Furthermore, flow-mediated dilation of the brachial artery

was not affected by the Glu298Asp polymorphism [20, 23], whereas Naber et al. demonstrated a higher coronary vascular resistance in 298Asp homozygotes compared to that of 298Glu homozygotes at rest [25]. These discrepancies might reflect the impact of interactions with environmental factors, such as smoking [2].

To explain this variability, Sofowora et al. put forward an interesting hypothesis. In spite of the observed reduction in urinary excretion of NO in 298Asp homozygotes, they could not demonstrate differences between 298Asp homozygotes and 298Glu homozygotes as to vascular responses to acetylcholine, glyceryl trinitrate or phenylephrine in a healthy population [32]. Since, in contrast to their results, 298Asp homozygous patients with ischemic heart disease were found to be more sensitive to the vasoconstrictive effects of phenylephrine, they suggested that the functional significance of the Glu298Asp polymorphism may only become manifest under conditions of endothelial dysfunction. In support of this possibility, Leeson et al. demonstrated that 298Asp homozygotes had significantly lower flow-mediated dilation than 298Glu homozygotes, only in smokers, a group known to have endothelial dysfunction [20]. Recently, smoking carriers of the 298Asp allele were also found to have an increased risk of coronary heart disease relative to non-smokers carrying two 298Glu alleles [19].

Endothelial dysfunction is an important characteristic of the metabolic syndrome. A study by Lteif et al. showed that insulin resistance and systolic blood pressure seem to be the most important determinants of endothelial dysfunction in patients with the metabolic syndrome [21]. Furthermore, high glucose levels are also known to determine endothelial dysfunction. A recent study showed that glucose downregulates *NOS3* mRNA and protein expression through regulation of mitochondrial production of reactive oxygen species (ROS) with subsequent activation of AP-1 [33]. The inhibitory effect of glucose on *NOS3* gene transcription was reversed by mutation of the AP-1 binding site in the promoter region. These findings indicate a mechanism by which hyperglycemia could increase the significance of *NOS3* promoter polymorphisms.

Thus, if we assume that the functional significance of *NOS3* polymorphisms depends on the presence of endothelial dysfunction, an explanation of our results may be that patients with the metabolic syndrome which carry the -949G, -716T or 298Asp alleles produce less NO than patients without the metabolic syndrome or carriers of the -949C, -716C or 298Glu alleles. Since the -949G/-716T haplotype showed a stronger associa-

tion with TVR than both polymorphisms individually, these alleles could even interact to reduce *NOS3* activity in patients with the metabolic syndrome. Further research has to be performed to test these hypotheses.

Although the variant alleles of the three *NOS3* polymorphisms did not increase the risk of restenosis in patients without the metabolic syndrome, the absolute rates of restenosis did not differ between subgroups with and without the metabolic syndrome. This implies that some other yet unknown factors may have contributed separately to the rate of restenosis in the group without the metabolic syndrome.

4.1. Limitations of the study

One of the limitations of our study is that we did not have waist circumference as per criteria of ATP-III. We therefore substituted waist circumference with variable of obesity as defined by BMI of $> 28.8 \text{ kg/m}^2$, this cutoff was equivalent to a waist circumference of 102 cm in a cross-sectional study and similar to the BMI value (28.2 kg/m^2) calculated in a regression of BMI on waist circumference in a large population of Scottish men [18,30].

In our study we lack data on *NOS3* activity or NO concentration. Abundant data is present on the functionality of the Glu298Asp polymorphism. However, little is known about a difference of *NOS3* activity between patients with and without the metabolic syndrome. We would like to promote research that compares *NOS3* activity and local NO concentrations between patients with the metabolic syndrome and controls having specific genotypes of the -949 A/G, -716 C/T and Glu298Asp polymorphisms.

5. Conclusions

In conclusion, we have demonstrated that the variant alleles of the -949 A/G, -716 C/T and Glu298Asp polymorphisms increase the risk of TVR in patients with the metabolic syndrome, but not in patients without the metabolic syndrome. We suggest that the metabolic alterations in these patients influence the functional significance of the three *NOS3* polymorphisms and increase the susceptibility of carriers of these variations in the *NOS3* gene to develop restenosis after PCI. Genotyping for these polymorphisms may lead to better risk stratification and more tailored therapy in patients with the metabolic syndrome to prevent TVR after PCI.

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